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TITLE OF THE INVENTION (280 characters max)

Thermo-Bio-Lithography

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THERMO-BIOLITHOGRAPHY: A TECHNIQUE FOR PATTERNING NUCLEIC ACIDS AND PROTEINS

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ABSTRACT

We describe a "biolithographic" technique, in which the unique properties of biopolymeric materials and the selective catalytic activities of enzymes are exploited for patterning surfaces under simple and bio-friendly conditions. We begin by coating a reactive film of the polysaccharide chitosan onto an inorganic surface (glass or silicon wafer). Chitosan's pH-responsive solubility facilitates film deposition, while the nucleophilic properties of this polysaccharide allow simple chemistries or biochemistries to be used to covalently attach species to the film. The thermally-responsive protein gelatin is then cast on top of the chitosan film, and the gelatin gel serves as a sacrificial "thermoresist". Pattern transfer is accomplished by applying a heated stamp to melt specific regions of the gelatin thermoresist and selectively-expose the underlying chitosan. Finally, molecules are conjugated to the exposed chitosan sub-layer and the sacrificial gelatin layer is removed (either by treating with warm water or protease). To demonstrate the concept, we patterned; a reactive dye (NHS-fluorescein), a model 20-base oligonucleotide (using standard glutaraldehyde coupling chemistries), and a model green fluorescent protein (GFP; using tyrosinase-initiated conjugation). Because gelatin can be applied and removed under mild conditions, sequential thermo-biolithographic steps can be performed without destroying previously-patterned biomacromolecules. These studies represent the first step toward exploiting nature's exquisite specificity for lithographic patterning.

INTRODUCTION

The incorporation of biological components (DNA, proteins, cells or tissue) into microfabricated devices offers considerable potential for biosensing and other applications.[1-14] However, the successful integration of biology and microfabrication requires methods that effectively accommodate the labile nature of the biological entities. We are investigating the use of biopolymers[15-18] and enzymes[19,20] for bio-fabrication. Biopolymers often have physicochemical properties that are stimuli-responsive under near-physiological conditions. Such stimuli-responsive properties suggest that "smart"

biopolymeric materials could be exploited for patterning under mild, "bio-friendly" conditions. Enzymes are selective catalysts that also function under mild conditions. Depolymerizing enzymes offer the potential for selectively removing biopolymeric films (e.g. for pattern transfer or resist removal) while conjugation enzymes offer the potential for covalently assembling biological components to fabricated systems. We refer to the use of biological materials and/or biological mechanisms for spatially-selective patterning as "biolithography"

Here we report an approach we term "thermo-biolithography" – the use of a thermally-responsive biological process for patterning. This approach is illustrated in Scheme 1. The first step of thermo-biolithography is to prepare the surface by coating the substrate with a reactive layer. In our studies, we use the pH-responsive and nucleophilic amino-polysaccharide chitosan as the reactive layer. A film of the thermally-responsive protein gelatin is then coated on top of the reactive layer to serve as a sacrificial barrier (thermoresist). As illustrated in Scheme 1, we next transfer a pattern to the surface using a heated stamp of suitable geometry. The gelatin resist layer melts in the regions where it comes in contact with the hot stamp and this exposes the underlying chitosan sub-layer. The pattern formed in the gelatin layer is a negative image of the stamp used. Patterning is followed by a conjugation step in which the species of interest (protein or nucleic acid) is conjugated to the exposed chitosan. Finally, Scheme 1 shows that the sacrificial gelatin layer is removed.

There are three goals for this study. First, we demonstrate the concept of thermo-biolithography by showing that gelatin can serve as a thermally-responsive resist. Second, we illustrate the utility of this approach by patterning a fluorescent dye, a model nucleic acid and a protein. Finally, we show that the mild conditions used for thermo-biolithography allow it to be performed sequentially to pattern multiple species of interest.

MATERIALS AND METHODS

Chitosan from crab shells (85% deacetylation and 370,000 molecular weight as reported by the manufacturer), gelatin (type A from porcine skin, approximately 175 Bloom),

phosphate buffered saline tablets (PBS), Tris-EDTA buffer (100X concentrate), sodium dodecyl sulfate, glutaraldehyde (grade I, 50 % w/w aqueous solution), urea (SigmaUltra grade), proteinase-K enzyme from *Tritirachium album* (47 U/mg), tyrosinase from mushroom (6680 U/mg), dimethylformamide (DMF), sodium hydroxide and ethanol (100 %), were all purchased from Sigma Chemicals. The fluorescent probes 5-(and 6-)–carboxyfluorescein succinimidyl ester (NHS-fluorescein) and 5-(and 6-)–carboxytetramethylrhodamine succinimidyl ester (NHS-rhodamine) were both purchased from Molecular Probes. Twice-HPLC-purified amine-terminated, fluorescently-labeled ssDNA of 20 bases from the *dnaK* genes of *Escherichia coli* were purchased from Gene Probe Technologies.

Details on the preparation of chitosan solutions as well as the fabrication of gold-coated wafers have been described elsewhere.[16,17] The protocol for the preparation of the fusion protein: Green Fluorescent Protein (GFP) with 6 histidine residues at its N-terminus and 5 tyrosine residues at its C-terminus $(His)_6$-GFP-$(Tyr)_5$ has also been described in earlier work.[19] To prepare 1 μg/ml of the GFP-solution, the concentrated protein solution was diluted using PBS. Gelatin solutions (15% w/w, pH = 7.5) were prepared by dissolving the required amount of gelatin in double-distilled water (DDW) at temperatures above 50°C. The pH was adjusted to 7.5 using small amounts of 1 M NaOH. PBS buffer was prepared by dissolving PBS tablets in DDW and adjusting the pH to 7.4. Tris-EDTA-SDS buffer was prepared by adding sodium dodecyl sulfate (SDS) to Tris-EDTA so as to have 0.5 % w/w SDS in the resulting solution. The 0.025 % w/w glutaraldehyde solution was prepared by diluting the 50 % w/w aqueous glutaraldehyde with PBS buffer. The 4 M urea solution was prepared by dissolving the urea pellets in DDW. Proteinase-K enzyme solution was prepared by dissolving 1 mg proteinase-K in 10 ml of Tris-EDTA-SDS buffer. Tyrosinase enzyme solution (2000 U/ml) was prepared by dissolving tyrosinase in DDW. NHS-fluorescein and NHS-rhodamine solutions were both prepared by first dissolving these compounds in 200 μL dimethylformamide and then adding 800 μL of ethanol. These fluorescein solutions were added drop-wise to PBS buffer to create the final reaction mixture.

Initial studies with NHS-fluorescein were performed using a glass microscope slide as the substrate. Nucleic acids and proteins were patterned using smaller substrates

to limit the consumption of the reagents. We selected silicon wafer 'chips' for these studies because of the ease of preparation of these substrates. Furthermore, these 'chips' were gold-coated to facilitate heat transfer during the thermal pattern transfer step. The chitosan sub-layer was prepared by spreading the acidic chitosan solution (2% w/w) onto the surface of the substrate and then immersing the substrate in base (1 M aqueous NaOH) to neutralize the chitosan. Chitosan is insoluble above a pH of about 6.5. The resulting sub-layer was washed extensively with DDW to remove traces of any salts formed during the neutralization and then air-dried. The gelatin thermoresist was cast on top of the chitosan sub-layer by spreading the gelatin solution (15% w/w at 50°C) and allowing it to air cool for 15 minutes. The stamps used for thermo-biolithography consisted of 0.6 mm-wide blades used either individually or as a pair separated by a spacer. The blades were heated on a hot plate to 50°C prior to stamping. Photographs were taken at the end of each study using a fluorescence stereomicroscope (MZ FLIII, Leica) with the GFP1 filter for green fluorescent protein, the GFP2 filter for fluorescein, or the 41004 TXRD filter for rhodamine. The GFP1 filter consisted of an excitation filter at 425 nm (band width of 60 nm) and an emission barrier filter at 480 nm. The GFP2 filter consisted of an excitation filter at 480 nm (band width of 40 nm) and an emission barrier filter at 510 nm. The 41004 TXRD filter consisted of an excitation filter at 560 nm (band width of 40 LP nm) and an emission barrier filter at 610 nm.

RESULTS AND DISCUSSION

1. Demonstrating the Concept of Thermo-biolithography for Surface Patterning

Our initial experiments were performed to demonstrate the concept of thermo-biolithography – that gelatin can be exploited as a temperature-responsive, sacrificial barrier (thermoresist) for the spatially-selective patterning of an underlying sub-layer. For this study, we used a glass slide, and coated it with a thin chitosan "reactive" sub-layer (estimated thickness was 1 μm or less) and then a thicker gelatin resist layer (~1 mm thick). The gelatin layer was allowed to gel by cooling at room temperature for 15 minutes. To transfer the pattern through the thermally-sensitive gelatin resist, we applied

a stamp heated to 50°C to the surface of the gelatin. The stamp consisted of two metallic blades clamped together and separated by a spacer (blades were approximately 0.6 mm wide and were separated by 4 mm). The heated stamp melted through the gelatin resist until it came in contact with the underlying chitosan sub-layer. Some gelatin in the vicinity of the stamp also melted while the chitosan sub-layer was unaffected by this thermal treatment. The stamp was held in place for several minutes to allow the melted gelatin to cool and reform a gel. The stamp was withdrawn from the gelatin layer just before this layer completely gelled. The timing for withdrawing the stamp was determined by manually probing the gelatin surface (with a spatula) to assess its elasticity. The timing of stamp withdrawal is important. If the stamp is withdrawn too soon - before gel formation - then the gelatin solution will be able to flow back and re-coat the sub-layer that was being exposed. If the stamp is withdrawn too late – after gel formation - then the gelatin adheres to the stamp and this resist is torn and partially delaminated during stamp withdrawal. This process selectively exposes the underlying sub-layer (in our case chitosan) and creates a pattern in the gelatin thermoresist layer that is a negative image of the applied stamp.

As illustrated in Scheme 1, the next step in our operation is to react the exposed sub-layer, while the gelatin resist protects the unexposed sub-layer. In our initial studies, we exposed our slide to a fluorescent dye with amine-reactive functionality. Specifically, we immersed the slide in a Petri dish containing 1.6 μg/ml NHS-fluorescein in 50 ml of PBS buffer (pH = 7.4) for 10 minutes. After reaction, the glass slide was recovered and rinsed extensively with water. Both the gelatin resist and the chitosan sub-layer were fluorescently-labeled by this amine-reactive reagent.

Scheme 1 shows that the final step in our operation is to remove the thermoresist. This was achieved by melting/dissolving away the gelatin layer by immersing the slide for 10 seconds in 1 liter of warm water (50°C). The time and temperature for resist removal must be adjusted based on gelatin's melting temperature which depends on gelatin's type and concentration. After removing the gelatin, the glass slide was rinsed with water and stored in PBS buffer.

Figure 1a shows a fluorescence photomicrograph of the glass slide after removal of the gelatin resist. As seen in Figure 1a, there are two fluorescent bands, each

approximately 0.6 mm in width spaced approximately 4 mm apart. These dimensions match the dimensions of the stamp used for the thermo-biolithography. To quantify patterning, we analyzed the fluorescence intensity profile from Figure 1a. Figure 1b shows a high fluorescence signal for the regions of the chitosan sub-layer that were exposed by thermo-biolithography. In contrast, the regions of the sub-layer that were protected by the gelatin thermoresist have low fluorescence intensity. The size and shape of the two peaks in Figure 1b indicate that the thermo-biolithographic pattern transfer is reproducible. In summary, Figure 1 demonstrates the concept of thermo-biolithography for the spatially-selective patterning of surfaces.

2. Nucleic Acid and Protein Patterning Using Thermo-biolithography

Nucleic acids and proteins are routinely used for biosensing[4,7,8] and often, it is desirable to pattern these biomacromolecules onto surfaces. Figure 2a illustrates our thermo-biolithographic approach for patterning nucleic acids. The substrate for these studies was a small rectangular "chip" (8 mm X 20 mm) that had been cut from a gold-coated silicon wafer. A chitosan sub-layer, and then a gelatin resist layer were coated onto the chip and pattern transfer was performed as described above. After pattern transfer, the exposed sub-layer was activated for nucleic acid conjugation by immersing the chip for 30 minutes in 5 ml of a PBS buffer solution containing 0.025% w/w glutaraldehyde. Glutaraldehyde is a homobifunctional amine-reactive crosslinking agent that has been used previously to couple amine-terminated single-stranded DNA oligonucleotides to chitosan.[18] After glutaraldehyde activation, the chip was rinsed extensively with PBS buffer to remove unreacted glutaraldehyde.

For nucleic acid coupling, the activated chip was placed in a 2 ml centrifuge tube with 1.5 ml of a PBS buffer containing 20 μg/ml of a fluorescently-labeled, amine-terminated oligonucleotide. The model oligonucleotide was a 20-base sequence from the *Escherichia coli dnaK* gene.[18] The coupling reaction was performed for 2 hours with mild agitation. After reaction, the chip was removed and rinsed extensively with PBS buffer. Figure 2b shows a fluorescence photomicrograph of this chip after reaction and rinsing.

As seen, the fluorescently-labeled oligonucleotide is coupled both to the exposed chitosan sub-layer and the gelatin resist.

As illustrated in Figure 2a, the next step is to remove the sacrificial gelatin resist. This resist could not be removed by simply dipping the chip in warm water because glutaraldehyde, which is used to activate the exposed chitosan sub-layer, also crosslinks gelatin and eliminates its ability to be melted or dissolved. To remove this crosslinked gelatin (protein) resist without destruction of either the conjugated nucleic acid or the chitosan polysaccharide sub-layer, we used a protease. Specifically, we placed the chip in a Petri dish with 5 ml of a Tris-EDTA-SDS buffer containing 100 μg/ml proteinase-K. To completely digest the gelatin, the chip was incubated in this solution with mild agitation for 6 hours at room temperature. After digestion, the chip was rinsed extensively with PBS buffer and examined using a fluorescence microscope. The fluorescence photomicrograph of Figure 2c shows two 0.6 mm-wide bands spaced 4 mm apart.

Image analysis of the fluorescence intensity of Figure 2c is shown in Figure 2d. As seen, higher fluorescence is observed in the regions where the chitosan sub-layer was exposed compared to the region protected by the gelatin thermoresist. To remove any physically bound oligonucleotides, we next immersed the chip in a 4 M urea solution for 1 hour at 50°C with mild agitation. Figures 2e and 2f show the fluorescence photomicrograph and corresponding fluorescence intensity profile for this chip after the urea wash. As can be seen, the urea wash had a relatively small effect on the images and the shapes of the intensity profiles of the patterned surface. This result provides evidence that the fluorescently-labeled oligonucleotide was covalently bonded to the exposed chitosan sub-layer. The similar size and shape of the two peaks in Figure 2f illustrates the reproducibility of thermo-biolithographic patterning.

As a control, we prepared the chip as illustrated in Figure 2a except the sample was not activated by glutaraldehyde. This "control" chip was also treated with fluorescently-labeled, amine terminated oligonucleotide solution. After removal of the gelatin with protease and performing the urea wash, the chip was examined using a fluorescence microscope. Figure 2g shows that no image is observed in the fluorescence photomicrograph of this control. This result indicates that glutaraldehyde activation is

necessary for coupling of the fluorescently-labeled, amine-terminated oligonucleotides. Overall, the results in Figure 2 demonstrate that thermo-biolithography can be combined with glutaraldehyde-based coupling chemistries for the patterning of oligonucleotides onto surfaces.

Scheme 2 shows that protein patterning was achieved using an enzyme-initiated method to conjugate proteins onto the exposed chitosan sub-layer. The substrate and pattern transfer steps were the same as those used for oligonucleotide patterning. Protein conjugation was initiated by the enzyme tyrosinase that converts accessible tyrosine residues of the protein into reactive *o*-quinone residues that can undergo coupling reactions with chitosan.[21] Our model protein was a fusion of green fluorescent protein with an N-terminus hexahistidine tag and a C-terminus pentatyrosine tag ($(His)_6$-GFP-$(Tyr)_5$).[19]

After thermo-biolithographic pattern transfer, the chip was dipped into 1.5 ml of a concentrated tyrosinase solution (2000 U/ml in a 2 ml centrifuge tube) for 30 minutes. This step served to "coat" the patterned chip with the tyrosinase coupling catalyst. In a second dip step, the chip was immersed in a 2 ml centrifuge tube containing 1.5 ml of a PBS buffer containing $(His)_6$-GFP-$(Tyr)_5$ (1 μg/ml). After a 2-hour incubation, the chip was recovered, washed extensively with PBS buffer, immersed in warm water (50°C for 60 seconds) to remove the gelatin thermoresist, and then extensively washed with PBS buffer. It should be noted that tyrosinase can react with the small number of tyrosine residues of gelatin, but these reactions do not prevent gelatin from melting.[21] The fluorescence photomicrograph of Figure 3a shows two 0.6 mm-wide fluorescent bands spaced 4 mm apart. The fluorescence intensity profile in Figure 3b further illustrates that tyrosinase can initiate the conjugation of GFP onto the exposed chitosan surface and that the patterning is reproducible.

As a control, we performed thermo-biolithography and dipped the chip in the GFP-containing solution as described above, except the chip was not dipped in tyrosinase. The fluorescence photomicrograph of this control is shown in Figure 3c and illustrates that very little fluorescence appears in this image. Thus, the results in Figure 3 demonstrate that proteins can be patterned onto chitosan surfaces using a combination of thermo-biolithography and tyrosinase-initiated conjugation.

3. Sequential Thermo-biolithography

The final goal of this work was to demonstrate that sequential thermo-biolithographic steps could be performed to spatially pattern different species onto a surface. In our initial study, we patterned two amine-reactive fluorescent dyes using the procedure illustrated in Figure 4a. In the first pattern transfer step, a single 0.6 mm wide line was transferred through the gelatin resist using one blade. After pattern transfer, this glass slide was reacted with NHS-fluorescein and then the gelatin resist was removed using warm water. As expected, a 0.6 mm-wide line was visible when the slide was examined using a fluorescence microscope (image not shown). For the second thermo-biolithographic step, the glass slide was once again coated with gelatin resist after which a 0.6 mm wide line was transferred through the gelatin resist. This second line was patterned to be parallel to the first. After this second pattern transfer step, the slide was placed in a Petri dish with 50 ml of PBS buffer (pH = 7.4) containing 1.6 μg/ml NHS-rhodamine. After allowing the reaction to proceed for 10 minutes, the slide was recovered, rinsed, and the thermoresist was removed with warm water. At the end of the experiment, the chitosan sub-layer had been patterned to have one fluorescein band and a second rhodamine band spaced approximately 4 mm apart.

To visualize the images transferred during this sequential patterning operation, fluorescence photomicrographs were obtained using two different filters – one for each dye. The upper photograph in Figure 4b shows the fluorescein band on the left, while the lower photomicrograph shows the rhodamine band on the right (the two bands could not be imaged simultaneously using our filter sets). Both bands in Figure 4b are 0.6 mm-wide. This result provides evidence that thermo-biolithography can be performed sequentially to pattern multiple compounds to chitosan.

A final study demonstrates that sequential thermo-biolithography can be performed with proteins. The schematic in Figure 5a shows that the first patterning was performed to create two parallel bands of $(His)_6$-GFP-$(Tyr)_5$. Pattern transfer, tyrosinase-initiated conjugation, and resist removal were achieved as described in the experiments

for Figure 3. Figure 5b shows the fluorescence photomicrograph and the fluorescence intensity profile after this first patterning.

The second thermo-biolithographic step was performed by coating gelatin over the chitosan sub-layer (including the region previously-patterned with GFP) and using a single blade to expose the chitosan sub-layer in a band perpendicular to the GFP bands. The chip was then reacted with 1 μg/ml of NHS-rhodamine, rinsed extensively with PBS buffer and then observed with the fluorescence microscope using two filters. Figure 5c shows a photomicrograph of the rhodamine band while Figure 5d shows a photomicrograph and the fluorescence intensity profile of the GFP bands. The comparison of Figures 5b and 5d indicates that there are no obvious differences in the GFP fluorescence images or intensity profiles before and after this second thermo-biolithographic step. Thus, the casting and subsequent removal of the gelatin thermoresist does not affect the fluorescence (and presumably structure) of previously-patterned GFP macromolecules.

CONCLUSIONS

Here we demonstrate that gelatin's thermally-responsive properties allow it to serve as an effective thermoresist, while chitosan's nucleophilic properties allow nucleic acids and proteins to be patterned using simple conjugation chemistries. To our knowledge, this is the first time that a thermally-responsive biopolymer has been exploited as a resist for patterning nucleic acids and proteins onto surfaces. Thermo-biolithography, as described in this work, has similarities to other patterning procedures. Like many printing operations,[22,23] a reactive sub-layer[24] is used for coupling the protein or nucleic acid to the substrate (chitosan was used in this study while poly-L-lysine is commonly used for micro-array pin printing). The gelatin thermoresist used here is analogous to the photoresists used in photolithography[25-30]- the resist protects certain regions during the conjugation step. Pattern transfer is accomplished in thermo-biolithography using a stamping operation that is analogous to microcontact printing (μCP).[31-36] Thermo-biolithography is also similar to molecular lithography where DNA serves as a template for metal coating and the RecA protein serves as a resist for this coating.[37] Finally,

proteases have been previously-reported as a method to remove a gelatin-based structural layer.[38]

It should stressed, that our goals were to demonstrate the concept of thermo-biolithography and illustrate its use for nucleic acid and protein patterning - we did not attempt to study the resolution limits of this technique. It seems likely that resolution could be significantly improved by better controlling how heat is transferred to the thermosresist. Rather than applying heat using a macroscopic stamp, stamps could be microfabricated, or heaters could be fabricated into the substrate (e.g. microhotplates).[39] Alternatively, heat could be applied in controlled photothermal processes (e.g. direct laser writing). Rapid, localized heating is commonly used in semiconductor processing operations such as graphoeptitaxy, zone melting recrystallization, and pulsed laser annealing.[40-43]

There are four reasons why we believe thermo-biolithography may offer benefits. First, patterning through thermo-biolithography is simple. Expensive equipment (e.g. pin printers) and facilities (e.g. clean rooms) are not needed for thermo-biolithography. The reactive chitosan sub-layer and the gelatin thermoresist are easily coated onto the substrate, while facile procedures can be used to conjugate nucleic acids or proteins. Second, pattern transfer and resist-removal are achieved under mild, bio-friendly conditions. This is illustrated by the results of Figure 5 that show GFP's fluorescence (and presumably structure) is unaffected by a subsequent lithographic step. Third, the use of biopolymers, enzymes (protease and tyrosinase), and aqueous solvents may provide safer and more environmentally friendly alternatives to existing fabrication methods while the products built using biopolymers should be biodegradable – a feature especially important for single-use applications. Finally, devices fabricated using gelatin and chitosan may have end-use applications in medicine (e.g. for drug delivery and artificial organs) since both biopolymers are expected to be biocompatible. Thus, thermo-biolithography is a simple and flexible alternative for patterning biomacromolecules.

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FIGURE LEGENDS

Figure 1. Demonstration of the concept of thermo-biolithography for surface patterning. (a) Fluorescence photomicrographs of patterned fluorescein bands using an excitation filter of 480 nm (bandwidth 40 nm), an emission barrier filter of 510 nm and exposure time of 90 seconds. (b) Fluorescence intensity profile of the patterned fluorescein surface. Width of each band ~ 0.6 mm, spacing between the bands ~ 4 mm.

Figure 2. Oligonucleotide patterning using thermo-biolithography. (a) Schematic of the steps involved in oligonucleotide patterning. (b) Fluorescence photomicrograph taken after reaction with amine-terminated, fluorescently-labeled oligonucleotides. (c) Fluorescence photomicrograph taken after protease treatment to remove gelatin resist. (d) Fluorescence intensity profile of the region photographed in Figure 2c. (e) Fluorescence photomicrograph taken after urea wash. (f) Fluorescence intensity profile of the region photographed in Figure 2e. (g) Fluorescence photomicrograph of a control that was not activated by glutaraldehyde. All photomicrographs were taken using an excitation filter of 480 nm (bandwidth 40 nm), an emission barrier filter of 510 nm and exposure time of 60 seconds. Width of each band ~ 0.6 mm, spacing between bands ~ 4 mm.

Figure 3. Protein patterning using thermo-biolithography. (a) Fluorescence photomicrograph of a GFP-patterned chitosan sub-layer. (b) Fluorescence intensity profile of the region photographed in Figure 3a. (c) Fluorescence photomicrograph of a control that lacked treatment with the tyrosinase conjugation catalyst. All fluorescence photomicrographs were taken using an excitation filter of 425 nm (bandwidth 60 nm), an emission barrier filter of 480 nm and exposure time of 15 seconds. Width of each band ~ 0.6 mm, spacing between bands ~ 4 mm.

Figure 4. Demonstration of sequential thermo-biolithography. (a) Schematic of the steps for sequential patterning. (b) Fluorescence photomicrographs taken after two patterning steps. The upper photomicrograph of the fluorescein band was taken using an excitation

filter of 480 nm (bandwidth 40 nm), an emission barrier filter of 510 nm and exposure time of 50 seconds. The lower photomicrograph of the rhodamine band was taken using an excitation filter of 560 nm (bandwidth 40 nm), an emission barrier filter of 610 nm and an exposure time of 50 seconds. Width of each band ~ 0.6 mm.

Figure 5. Sequential thermo-biolithography with a patterned protein. (a) Schematic of the two patterning steps. (b) Fluorescence photomicrograph and fluorescence intensity profile taken after the first patterning step that generates two parallel bands of GFP. (c) Fluorescence photomicrograph of the rhodamine band taken after the second patterning step. (d) Fluorescence photomicrograph and fluorescence intensity profile of the GFP bands taken after the second patterning step. GFP images were obtained using an excitation filter of 425 nm (bandwidth 60 nm), an emission barrier filter of 480 nm and an exposure time of 10 seconds. The rhodamine band was observed using an excitation filter of 560 nm (bandwidth 40 nm), an emission barrier filter of 610 nm and an exposure time of 30 seconds.

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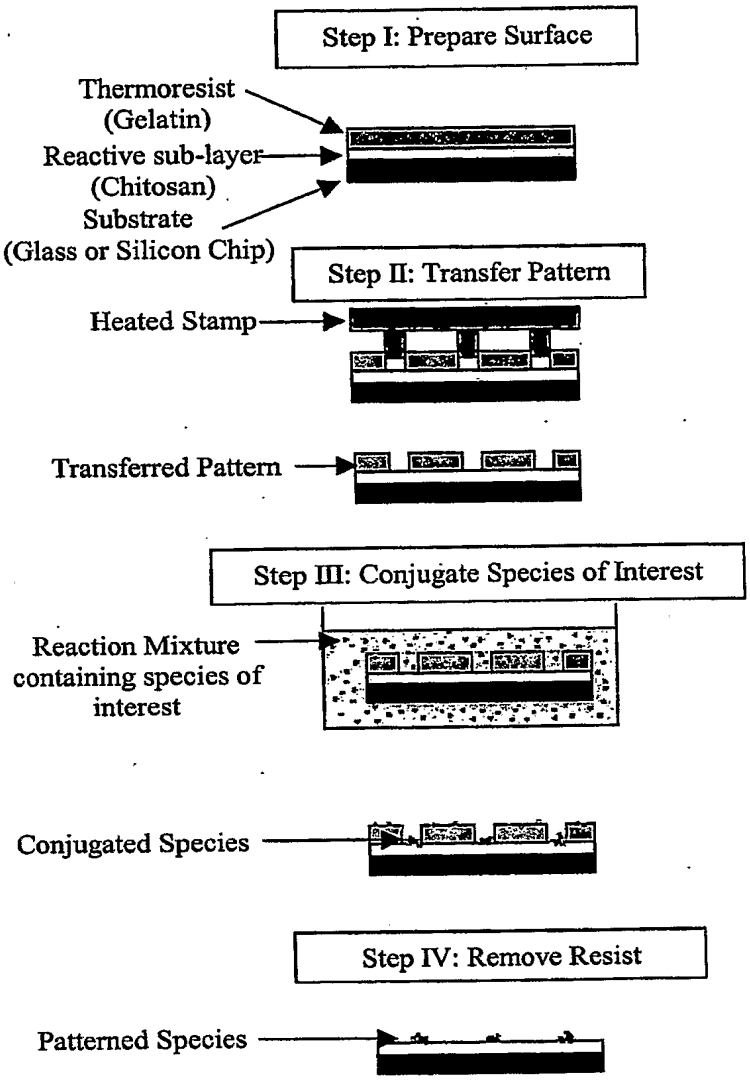


Scheme 1

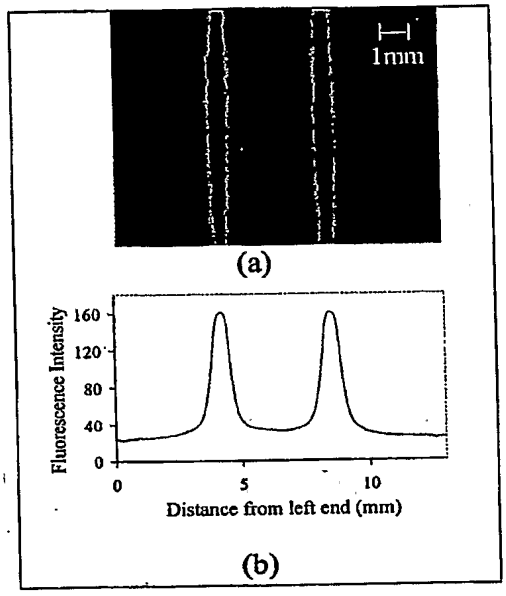


Figure 1

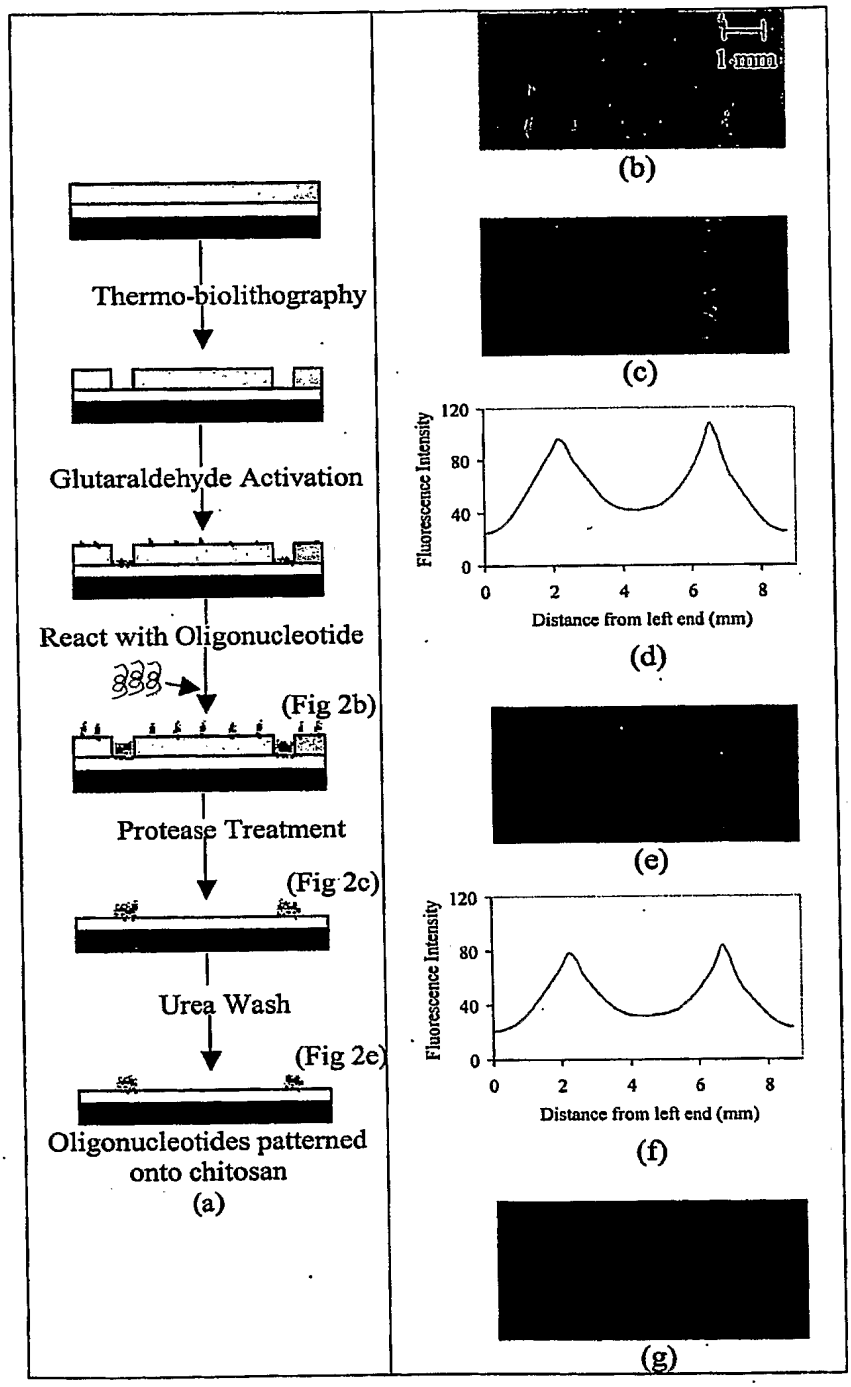
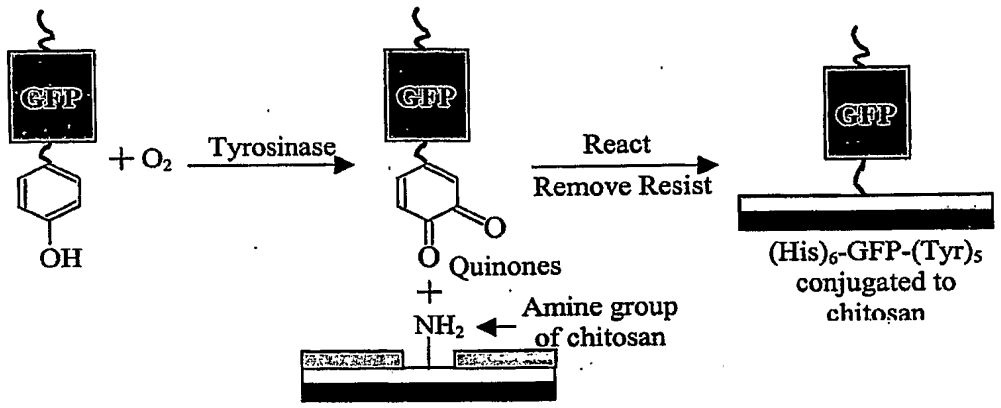


Figure 2



Scheme 2

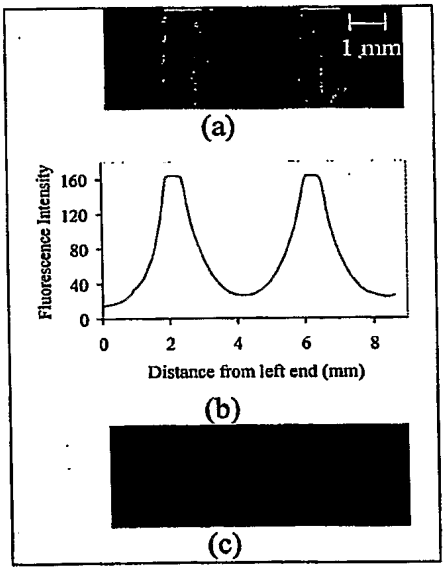


Figure 3

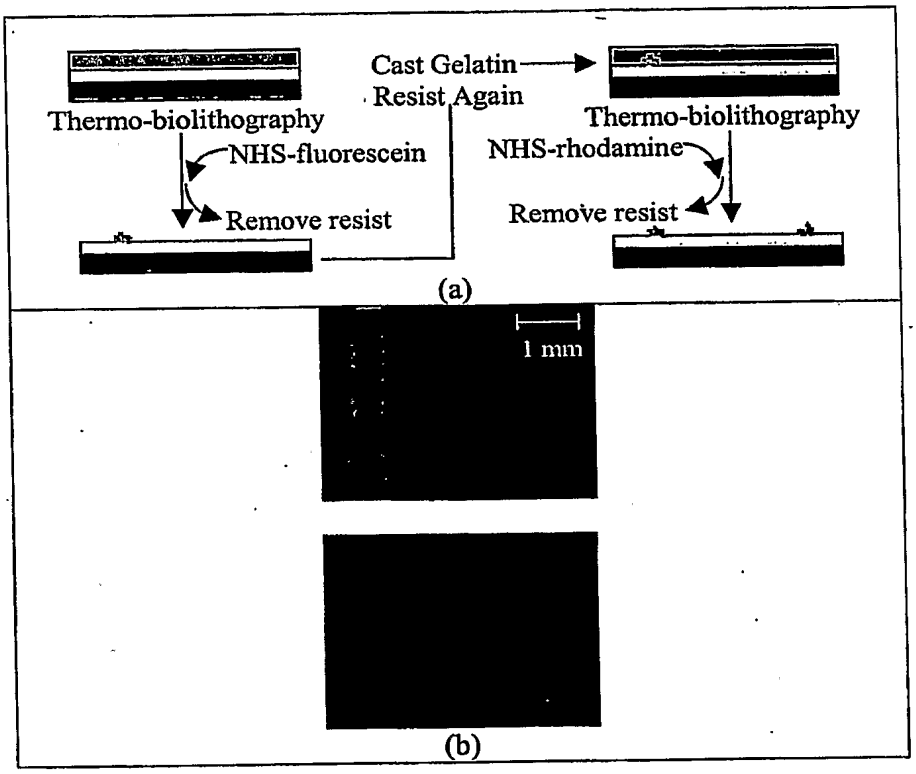

Cast Gelatin
Resist Again
Thermo-biolithography
NHS-fluorescein
Remove resist
Thermo-biolithography
NHS-rhodamine
Remove resist
(a)
1 mm
(b)

Figure 4

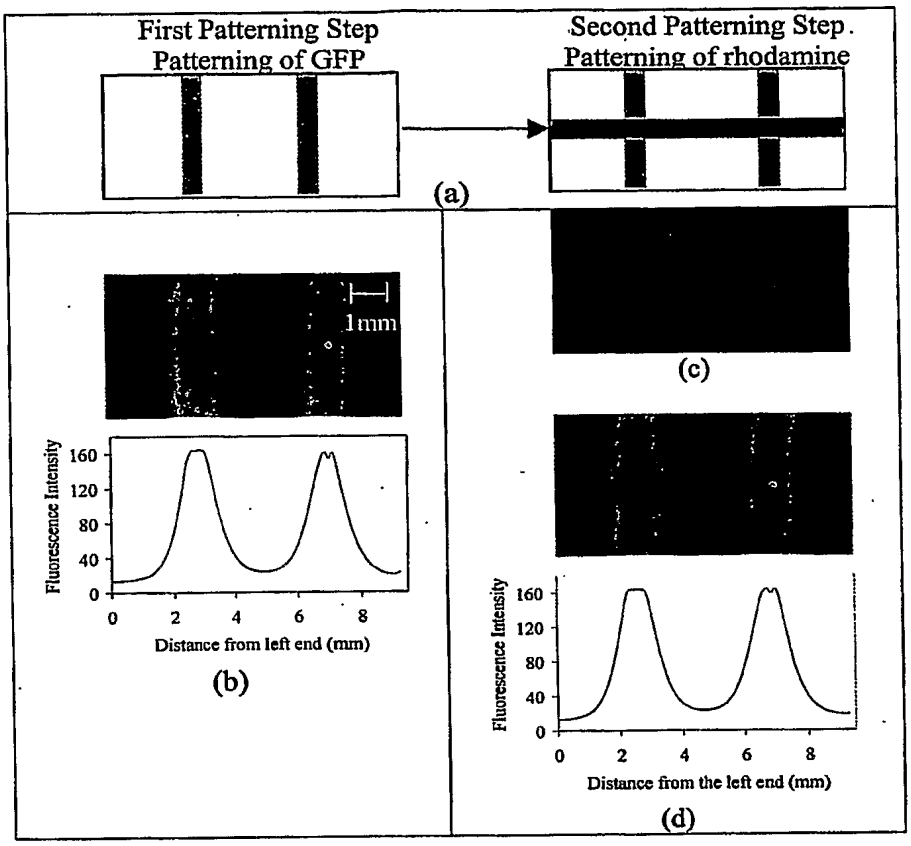


Figure 5